2.0 *IN VITRO* ER COMPETITIVE BINDING ASSAY METHODS

2.1 Introduction

The basic procedures to measure test substance binding to the ER were developed between 1965 and the early 1970s (Clark and Gorski, 1969). Cells containing an ER, semi-purified ER, or cytosolic fractions from cells containing an ER (typically from the uterus) are treated with sufficient amounts of radiolabeled reference estrogen (generally 17β-estradiol) to saturate all of the ER binding sites. Following this treatment, the cells, proteins, or cellular extracts are challenged with the test substance, and the amount of radioactive reference estrogen remaining bound to the ER is measured by scintillation counting. The amount of bound radiolabeled reference estrogen is a function of the receptor-binding capacity of the test substance and the test substance concentration. Recently, a technique known as FP, in which a fluorescent estrogen molecule replaces the radiolabeled reference estrogen has been developed. In this assay, changes in the polarization of light are measured rather than scintillation counting of the amount of ER-bound radiolabeled reference estrogen.

Results from these competition assays are expressed as the K_i or as the IC_{50}. The K_i is a function of the affinity of the test substance and the radiolabeled reference estrogen for the ER. Despite the fact that the IC_{50} is very sensitive to experimental conditions while the K_i is less sensitive to these conditions, the majority of investigators present their data as IC_{50} values. This may be due to the fact that the most commonly used approach for comparing data within and between laboratories is the RBA, which is based on IC_{50} values.

The basic procedure proposed by Hähnel (1971) and Korenman (1970), among others, to measure ER binding has been modified over the years by numerous investigators. Common modifications include the source of the ER, the exposure duration and temperature, the reference estrogen, and the adsorbent used to separate the bound, radiolabeled estrogen from unbound molecules. For the purpose of summarizing the available ER binding assay approaches used by different investigators (**Appendix A**), the various protocols have been sorted according to whether they were performed with intact cells, a cell cytosol preparation, or with semi-purified preparations of human or rat ERα or ERβ proteins. The protocols using cytosol have been further categorized according to the source of the cytosol (i.e., rat, mouse, or rabbit uterine

cytosol and cytosol from MCF-7 cells). The data generated from studies using a glutathione construct of the ligand binding domain (def) of the ERα from humans and mouse, and the ER from a lizard (anole), chicken, and rainbow trout were categorized separately.

The first step in an ER binding assay is to determine the K_d of the reference estrogen (e.g., 17β-estradiol) to the ER preparation used in the assay. The purpose of determining the K_d for each ER assay system is to demonstrate that the assay system is valid (e.g., a finite number of high affinity receptors are saturated with ligand) and to optimize the system with respect to receptor and ligand concentration. The K_d is determined in a saturation binding experiment that involves adding increasing concentrations of the radiolabeled reference estrogen to the cells/cytosol and measuring the amount that binds to the ER (Motulsky, 1995). To calculate specific binding of the radiolabeled reference estrogen to the ER, nonspecific binding (i.e., binding to sites other than ER) is measured at each radioligand concentration by the addition of a nonlabeled estrogen at a concentration that occupies all available receptors. The nonspecific binding is subtracted from the total binding (in the absence of nonlabeled compound) of the radiolabeled reference estrogen (Motulsky, 1995). The amount of radioligand specifically bound depends on the number (concentration) of receptors in the preparation. Free and bound radiolabeled ligands are separated by the addition of a nonreactive absorbent, such as dextran charcoal or HAP. The ER, the bound radiolabeled reference estrogen, and other proteins in the reaction mix bind to the absorbent, while the displaced radiolabeled reference estrogen remains in the supernatant. The mixture is centrifuged and the amount of ER-bound radiolabeled reference estrogen in the pellet is measured. The K_d of the reference estrogen, which reflects its affinity for the specific ER preparation, can then be calculated. The K_d is used to determine the appropriate concentration of the reference estrogen to be used in the competitive binding assay.

Because the largest proportion of the published data was derived from studies using uterine cytosol from rats and mice, a general guideline for this method is described first. This guideline is followed with less detailed descriptions of other assays used to measure ER binding. In addition, copies of protocols were requested from investigators using the different *in vitro* ER binding assays; copies of the protocols received for public distribution are provided in **Appendix B**.

2.2 General Overview of *In Vitro* Assays Used To Measure Competitive ER Binding

The primary purpose of most *in vitro* ER binding studies was to investigate the nature of the binding process and the kinetics of the reaction, and to identify which molecular moieties enhanced or inhibited binding to the ER. Thus, most studies were not conducted to specifically identify endocrine disruptors. The publications presenting the results of relevant studies provided various levels of detail on the methods used, ranging from highly specific protocols to a simple listing of the ER source and the identity of the test substances.

A general protocol using uterine cytosol from rats and mice is described below, followed by less detailed descriptions of other assays used to measure ER binding. The majority of the *in vitro* ER binding studies considered for this BRD used 17β-estradiol as the reference estrogen and, thus, this estrogen is included in the general protocols described in the following sections.

2.2.1 Mammalian Uterine Cytosol (Rat, Mouse, Rabbit) as the ER Source

Uterine cytosol is prepared by homogenizing the uterus in cold Tris buffer in a 1:10 ratio of tissue to buffer. The homogenate is centrifuged for 10 minutes at 2,500 x g at 4°C and the pellet containing cell debris is discarded. Next, the supernatant is centrifuged at 105,000 x g for 60 minutes at 4°C to pellet organelles and the cell cytosol supernatant containing the ER is stored at -70°C. Cytosolic protein concentration is determined using conventional methods.

To determine the K_d of 17β-estradiol, radiolabeled (i.e., with ^3H) 17β-estradiol at concentrations ranging from $1.0x10^{-8}$ to $3.3x10^{-11}$ M in buffer is added to aliquots of cytosol. Nonspecific binding of the radiolabeled 17β-estradiol is measured at each concentration by the addition of nonlabeled 17β-estradiol at a concentration that occupies all available receptors. Specific binding to the ER is then calculated at each concentration by subtracting nonspecific 17β-estradiol binding from the total binding of 17β-estradiol. After incubation and separation of bound and unbound radiolabeled 17β-estradiol, the amount of radiolabeled 17β-estradiol bound to the ER is measured. Specific binding data from saturation assays are usually analyzed to obtain the number of binding sites in a cytosolic preparation, B_{max}, and the K_d by nonlinear

regression using log concentration of radiolabeled 17β-estradiol as the independent variable (Motulsky, 1995).

The saturation binding curve of radiolabeled 17β-estradiol can also be analyzed using a linear Scatchard analysis (Scatchard, 1949) with specific binding on the abscissa (usually labeled "Bound") and the ratio of specific binding of 17β-estradiol to free 17β-estradiol (usually labeled "Bound/Free") on the ordinate. In these plots, B_{max} is the x-intercept and K_d is the negative reciprocal of the slope. However, the Scatchard plot is not the most accurate technique to use for analysis because the data is transformed to make a linear graph that is then analyzed by linear regression, and transformation of the data distorts the experimental error. Linear regression analysis assumes that the scatter of points around a line follows a Gaussian distribution so that the standard deviation is the same at every value of X. However, this is not true with transformed data. Secondly, a Scatchard transformation alters the relationship between the "Bound" (X) and the "Bound/Free" (Y) ratio. This is because the value of X is used to calculate the value of Y and this calculation violates the assumptions of linear regression. Thus, the Scatchard values for B_{max} and K_d are often further from their true values than if they had been calculated using nonlinear regression.

To measure competitive binding, radiolabeled 17β-estradiol, at a concentration that approximates the K_d, is added to tubes containing aliquots of the cytosol. A range of concentrations of the test substance in solvent, usually ethanol or unlabeled 17β-estradiol, is added. Nonspecific binding of radiolabeled 17β-estradiol to the receptor is measured by using a 100-fold molar excess of unlabeled 17β-estradiol. Following incubation of the mixture, the displaced radiolabeled 17β-estradiol is separated from the receptor-bound radiolabeled 17β-estradiol using an absorbent, such as dextran charcoal or HAP. The radiolabeled 17β-estradiol-ER complex and the test substance-ER complex bind to the absorbent, and the unbound radiolabeled 17β-estradiol and test substance are removed by extensive washing of the absorbent. After centrifugation, the bound radiolabeled 17β-estradiol in the pellet is extracted with ethanol and the concentration of radiolabel is determined by scintillation counting. Specific binding is calculated by subtracting the amount of nonspecific binding from each sample evaluated in the assay. Data for the binding of the radiolabeled 17β-estradiol and its displacement by each test substance are plotted as the

percentage of radiolabeled 17β-estradiol bound versus the molar concentration of competing test substance. For a substance with high affinity for the receptor, the upper plateau of the curve

$$\text{Specific binding} = \frac{B_{max} \times [\text{Free radiolabeled } 17\beta\text{ - estradiol}]}{K_d + [\text{Free radiolabeled } 17\beta\text{ - estradiol}]}$$

correlates with maximal receptor binding in the absence of the test substance, and the bottom of the curve is the nonspecific binding. The concentration of the test substance that produces radiolabeled 17β-estradiol binding half way between the upper and lower plateaus is the IC_{50}. Estimates of the IC_{50} can be determined using appropriate statistical software.

The K_i, which reflects the affinity of the test substance for the ER, can be calculated from the IC_{50} value using the equation of Cheng and Prusoff (1973):

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Radiolabeled } 17\beta\text{ - estradiol}]}{K_d}}$$

The RBA value for each competing test substance is calculated by using the following equation:

$$\text{RBA} = \frac{IC_{50} \text{ for } 17\beta\text{ - estradiol}}{IC_{50} \text{ for test substance}} \times 100$$

2.2.2 MCF-7 Cells and MCF-7 Cell-derived Cytosol

2.2.2.1 Intact MCF-7 Cells as ER Source

A number of cell lines inherently contain ER. The cell line most widely used for evaluating ER binding is the human breast adenocarcinoma cell line MCF-7. These cells are maintained in standard growth medium. Prior to their use in ER binding assays, the cells are grown for one to two days in medium containing charcoal-stripped serum. The purpose of charcoal stripping is to remove residual estrogenic substances that may competitively interfere with the binding of reference estrogens and test substances to the receptor.

For testing, intact cells are washed and treated with the radiolabeled 17β-estradiol in serum-free minimal medium. Unlabeled test substances, including 17β-estradiol, are added to the cells under non-growth conditions. Following incubation, the unbound test substance and reference estrogen are removed by washing the cells with ethanol. Scintillation counting is used to determine the extent of binding of the labeled reference estrogen. The amount of radiolabeled reference estrogen displaced by the test substance is used as the measure of its binding affinity for the ER.

2.2.2.2 MCF-7 Cytosol

A cell-free (cytosolic) extract of MCF-7 cells, which is prepared in a similar manner to cytosolic extracts from the rodent uterus, has been used as a source of ER. Cultured MCF-7 cells are harvested, homogenized to disrupt the cell membranes, and centrifuged to separate the nuclear debris and organelles from the cytosol. Generally, the assay is performed as outlined for the uterine cytosol assay.

2.2.3 Semi-Purified ERα and ERβ

In the past few years, researchers have recognized the advantages of using molecular techniques to isolate the ER from mammalian tissues or to clone the DNA coding for the receptor into a plasmid, transfect a cell with the plasmid, and express the ER in a cell. The protein can be isolated and purified, or the cellular extract can be processed such that a semi-purified ER is obtained. Transfected cells or other cell lines with DNA transcripts from different species that code for the complete ER or for selected domains of the ER have been constructed.

Different approaches have been used to produce semi-purified ERα and ERβ proteins for use as receptors to measure binding. To produce these proteins, the cDNA of the corresponding ER genes are cloned into a baculovirus or a transfer vector. The recombinant baculovirus vector is amplified and used to infect insect Sf9 cells (Bolger et al., 1998). Two days after infection, the cells are harvested and nuclei are isolated. A nuclear extract is made with buffer and the concentrations of ER proteins determined based on the specific binding of 17β-estradiol to the receptors in solution (Kuiper et al., 1998). The semi-purified ER preparation compares favorably to the ER isolated from tissue preparations with respect to size, immunogenicity, hormone

binding characteristics, phosphorylation state, and DNA interactions in gel shift assays. This ER also interacts normally with its DNA response element (Cheskis et al., 1997; Ozers et al., 1997).

In some approaches, the cDNA of the ER protein is transcribed, whereas in others, only the cDNA coding for the ligand-binding domain of the ER protein is specifically excised and cloned, and the partial protein is expressed for use in the assay. Another approach has been the use of a rabbit cell expression lysate to produce the ERα and ERβ proteins. The semi-purified ER proteins are produced by cloning the receptor genes into a plasmid, followed by the synthesis of the protein using the TnT-coupled reticulocyte lysate system with T7-RNA polymerase. Aliquots of the translation reaction mixture are used in the competitive binding assay (Kuiper et al., 1997).

2.2.3.1 Solid Phase Ligand Binding Assay using ScintiStrip™ (Kuiper at al., 1998)

The wells of ScintiStrip™ microtiter plates have scintillation fluors incorporated into the plastic. Signal detection is based on the premise that tritium (^{3}H) is a weak β emitter and low energy electrons have a short range in solution; the tritiated molecules, binding to the solid support containing the fluor, will trigger a response. The assay is performed by binding the ERα and ERβ proteins to the plastic support, followed by the addition of radiolabeled 17β-estradiol and the test substance. Scintillation counting of the wells will detect only the radiolabeled 17β-estradiol that remains bound to the ER, whereas radiolabeled 17β-estradiol displaced from the ER by the test substance will not be detected.

2.2.4 GST-ERdef Fusion Proteins

GST-ERdef fusion proteins contain only the ligand binding domain (known as the def domains) of the ER fused to glutathione-*S*-transferase (GST). The def domains have been transcribed and translated for use in measuring the ER-binding of 17β-estradiol and other substances. This approach has been used to prepare the partially purified binding domains of the ER protein from the lizard (anole), chicken, and rainbow trout, and the ERα from human and mouse (Matthews et al., 2000; Matthews and Zacharewski, 2000).

Essentially, assays using GST-ERdef proteins are performed as described above for cytosol except incubations are in 1 mL glass tubes arranged in a 96-well format. Bound radiolabeled 17β-estradiol is separated from free radiolabeled 17β-estradiol using a 96-well filter plate and vacuum pump harvester. The filter plates containing the protein are washed with buffer and the plates are allowed to dry under continuous suction. After drying, the undersides of the filter plates are sealed and scintillation cocktail is added to each well. Bound radiolabeled 17β-estradiol is measured using a scintillation counter. Nonspecific binding of 17β-estradiol is determined in the presence of a 400-fold excess of unlabeled 17β-estradiol (Matthews and Zacharewski, 2000).

2.2.5 Fluorescent Polarization (FP)

2.2.5.1 Theory of FP

FP is a technique that can detect molecular interactions by monitoring changes in the size of fluorescently labeled or inherently fluorescent molecules (Dandliker et al., 1981; Checovich et al., 1995; Jameson and Sawyer, 1995; Lundblad et al., 1996). When a fluorescent molecule binds to another molecule, its speed of rotation changes. This change in speed or tumbling rate can be quantified by FP. When a solution of fluorescent molecules is excited by plane-polarized light, those molecules parallel to the plane become excited. If the molecules remain stationary during the period of excitation (4 nanoseconds for fluorescein), the emitted light remains highly polarized. However, if the molecules tumble during the period of excitation, the emitted light will be random or depolarized. An increase in the volume or conformation of a fluorescent molecule (e.g., through its binding to a receptor or antibody) or a decrease in its conformation or molecular volume (due to dissociation or enzymatic degradation) can be directly measured by FP. The observed value is a weighted average of the polarization values of the individual bound and free fluorescent molecules, and is therefore a direct measure of the fraction bound. The concentration of the bound ligand is derived from the polarization value, and the resultant bound versus free isotherm is analyzed in a similar manner to the graph generated by conventional techniques for radioactivity (Dandliker et al., 1981; Checovich et al., 1995; Jameson and Sawyer, 1995).

For the FP assay, purified, full-length hERα or hERβ and an intrinsically fluorescent nonsteroidal estrogen (Fluormone ES1; FES1), which binds to the hER with high affinity, are used (Bolger et al., 1998). This particular fluorescent estrogen was developed by Katzenellenbogen and colleagues (Hwang et al., 1992).

In the competitive binding assay, substances are tested for their ability to displace the fluorescent ligand FES1 from an ER-FES1 complex. The large ER-FES1 complex tumbles slowly and therefore has a high anisotropy value. As increasing concentrations of a competing ligand displace the FES1 from the complex, the free FES1 molecules tumble more rapidly and have a lower anisotropy value. As more FES1 molecules are displaced from the complex, the measured anisotropy approaches the free anisotropy value. The measured anisotropy is a weighted average of the bound and free FES1 molecules.

2.2.5.2 Conduct of the Assay

As described for the other assays, a binding constant of reference estrogen, in this case FES1, to the receptor must be determined. This approach ensures that a saturating concentration of FES1 is used in the competitive binding assay. The receptor is serially diluted and the same concentration of FES1 is added to each tube. After incubation at room temperature, the fluorescence anisotropy of each tube is measured with a 360 nm excitation filter and a 530 nm emission filter. The anisotropy at each ER concentration is converted to the fraction of ligand bound using the following equation:

$$F_b = \frac{A - A_f}{A_b - A_f}$$

where F_b is the fraction of ligand bound, A is observed anisotropy, and A_b and A_f are anisotropy values of the bound and free ligand, respectively.

Bound ER (ER_b) is assumed to be equal to bound ligand (L_b), and therefore determined by multiplying F_b by the total ligand concentration (L_t). Free ER (ER_f) is calculated by subtracting ER_b from the total ER in the assay (ER_t). The equilibrium binding constant, K_d, is calculated from the ER-bound versus ER-free isotherm using a nonlinear least-square curve fitting program.

In performing the competitive binding assay, aliquots of the serially diluted test compound are added to known concentrations of hERα and FES1. Negative controls containing hERα + FES1 (equivalent to 0% inhibition), and positive controls containing free FES1 (equivalent to 100% inhibition), in the absence of competitor, are included in each run. Varying concentrations of the competitive ligand are added to tubes containing the same concentrations of hERα and FES1. After incubation at room temperature, the anisotropy value in each tube is measured. The anisotropy values are converted to percent inhibition using the following formula:

$$I\% = \frac{A_o - A}{A_o - A_{100}} \times 100$$

where A_o, A_{100}, and A are the percent inhibition, A_o at 0% inhibition, A_{100} at 100% inhibition, and the observed A value, respectively.

Polarization values are converted to percent inhibition to normalize day-to-day differences in the starting 0% inhibition polarization values. The percent inhibition versus competitor concentration curves is analyzed by nonlinear least-squares curve fitting to yield an IC_{50} value. IC_{50} values are converted to an RBA value using 17β-estradiol as a standard; the RBA value using 17β-estradiol is set to 100.

2.2.6 Permutations of the Assays as Described in the Literature

Irrespective of source of the ER used in a particular study, the protocols vary from laboratory to laboratory. Some of these variations are in response to the differing properties of the ER preparations used, or because of various questions the studies were designed to address. The permutations in the protocols used by each laboratory for each source of ER are summarized in **Appendix A**.